The maternal hormone in the male brain: sexually dimorphic distribution of prolactin signalling in the mouse brain

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ABSTRACT

Research of the central actions of prolactin (PRL) is virtually restricted to females, but this hormone has also documented roles in male physiology and behaviour. Here, we provide the first description of the pattern of PRL-derived signalling in the male mouse brain, employing the immunostaining of phosphorylated signal transducer and activator of transcription 5 (pSTAT5) after exogenous PRL administration. Next, we explore possible sexual dimorphism by comparing pSTAT5 immunoreactivity in PRL-supplemented males and females. We also assess the role of testosterone in the regulation of central prolactin signalling in males by comparing intact with castrated PRL-supplemented males.

Prolactin-supplemented males displayed a widespread pattern of pSTAT5 immunoreactivity, restricted to brain centres showing expression of the PRL receptor. Immunoreactivity for pSTAT5 was present in several nuclei of the preoptic, anterior and tuberal hypothalamus, as well as in the septofimbrial nucleus or posterodorsal medial amygdala of the telencephalon. Conversely, non-supplemented control males were virtually devoid of pSTAT5-immunoreactivity, suggesting that central PRL actions in males are limited to situations concurrent with substantial hypophyseal PRL release (e.g. stress or mating). Furthermore, comparison of PRL-supplemented males and females revealed a significant, female-biased sexual dimorphism, supporting the view that PRL has a preeminent role in female physiology and behaviour. Finally, in males, castration significantly reduced pSTAT5 immunoreactivity in some structures, including the paraventricular and ventromedial hypothalamic nuclei and the septofimbrial region, thus indicating a region-specific regulatory role of testosterone over central PRL signalling.

Précis

We map prolactin signalling in the male brain using pSTAT5-immunostaining after prolactin administration. We show that prolactin signalling is both sexually dimorphic and modulated by testosterone.

ABBREVIATIONS

3V	third ventricle
AAD	anterior amygdaloid area, dorsal part
AC/ADP	anterior commissural nucleus/anterodorsal preoptic nucleus region
ac	anterior commissure
Acb	nucleus accumbens
ACo	anterior cortical amygdaloid nucleus
AHi	amygdalohippocampal area
AHP	anterior hypothalamic area, posterior part
Aq	aqueduct (Sylvius)
Arc	arcuate hypothalamic nucleus
AVP	arginine-vasopressin
AVPe	anteroventral periventricular nucleus
Bar	Barrington´s nucleus
BLA	basolateral amygdaloid nucleus, anterior part
BLV	basolateral amygdaloid nucleus, ventral part
BMP	basomedial amygdaloid nucleus, posterior part
BSTIA	bed nucleus of the stria terminalis, intraamygdaloid division
BSTLD	bed nucleus of the stria terminalis, lateral division, dorsal part
BSTLP	bed nucleus of the stria terminalis, lateral division, posterior part
BSTLV	bed nucleus of the stria terminalis, lateral division, ventral part
BSTMA	bed nucleus of the stria terminalis, medial division, anterior part
BSTMV	bed nucleus of the stria terminalis, medial division, ventral part
BSTMPI	bed nucleus of the stria terminalis, medial division, posterointermediate part
BSTMPL	bed nucleus of the stria terminalis, medial division, posterolateral part
BSTMPM	bed nucleus of the stria terminalis, medial division, posteromedial part
cc	corpus callosum

1	Ce	central amygdala
2	CeC	central amygdaloid nucleus, capsular part
3	CeL	central amygdaloid nucleus, lateral division
4	CeM	central amygdaloid nucleus, medial division
5	CPu	caudate putamen (striatum)
6	CRF	corticotropin-releasing factor
7	CxA	cortex-amygdala transition zone
8	D3V	dorsal third ventricle
9	DEn	dorsal endopiriform nucleus
10	Dk	nucleus of Darkschewitsch
11	DLG	dorsal lateral geniculate nucleus
12	DLPAG	dorsolateral periaqueductal grey
13	DM	dorsomedial hypothalamic nucleus
14	DMPAG	dorsomedial periaqueductal grey
15	DMTg	dorsomedial tegmental area
16	DpG	deep grey layer of the superior colliculus
17	DpMe	deep mesencephalic nucleus
18	DpWh	deep white layer of the superior colliculus
19	DR	dorsal raphe nucleus
20	DTg	dorsal tegmental nucleus
21	f	fornix
22	fi	fimbria of the hippocampus
23	HDB	nucleus of the horizontal limb of the diagonal band
24	I	intercalated nuclei of the amygdala
25	ic	internal capsule
26	IF	interfascicular nucleus
27	InC	interstitial nucleus of Cajal
28	InG	intermediate grey layer of the superior colliculus
29	InWh	intermediate white layer of the superior colliculus

IPAC	interstitial nucleus of the posterior limb of the anterior commissure
La	lateral amygdaloid nucleus
LA	lateroanterior hypothalamic nucleus
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus
LGP	lateral globus pallidus
LH	lateral hypothalamic area
LHb	lateral habenular nucleus
lo	lateral olfactory tract
LOT	nucleus of the lateral olfactory tract
LPAG	lateral periaqueductal grey
LPBC	lateral parabrachial nucleus, central part
LPBD	lateral parabrachial nucleus, dorsal part
LPBE	lateral parabrachial nucleus, external part
LPBI	lateral parabrachial nucleus, internal part
LPBV	lateral parabrachial nucleus, ventral part
LSD	lateral septal nucleus, dorsal part
LSI	lateral septal nucleus, intermediate part
LSV	lateral septal nucleus, ventral part
LV	lateral ventricle
MCLH	magnocellular nucleus of the lateral hypothalamus
mcp	middle cerebellar peduncle
MCPO	magnocellular preoptic nucleus
ME	median eminence
MeA	medial amygdaloid nucleus, anterior part
MePD	medial amygdaloid nucleus, posterodorsal part
MePV	medial amygdaloid nucleus, posteroventral part
MGD	medial geniculate nucleus, dorsal part
MGM	medial geniculate nucleus, medial part

1	MGP	medial globus pallidus
2	MGV	medial geniculate nucleus, ventral part
3	MHb	medial habenular nucleus
4	ml	medial lemniscus
5	mlf	medial longitudinal fasciculus
6	MnPO	median preoptic nucleus
7	MnR	median raphe nucleus
8	MPA	medial preoptic area
9	MPB	medial parabrachial nucleus
10	MPBE	medial parabrachial nucleus, external part
11	MPO	medial preoptic nucleus
12	MS	medial septal nucleus
13	mt	mammilothalamic tract
14	MTu	medial tuberal nucleus
15	MZMGV	marginal zone of the medial geniculate
16	opt	optic tract
17	OT	oxytocin
18	ox	optic chiasm
19	Pa	paraventricular nucleus
20	PaLM	paraventricular nucleus, lateral magnocellular part
21	PaMM	paraventricular nucleus, medial magnocellular part
22	PaPO	paraventricular nucleus, posterior part
23	PaV	paraventricular nucleus, ventral part
24	PAG	periaqueductal greyPe periventricular hypothalamic nucleus
25	PeF	perifornical nucleus
26	PH	posterior hypothalamic area
27	PIL	posterior intralaminar thalamic nucleus
28	Pir	piriform cortex
29	PLCo	posterolateral cortical amygdaloid nucleus

1	PnC	pontine reticular nucleus, caudal part
2	PnO	pontine reticular nucleus, oral part
3	PnR	pontine raphe nucleus
4	PnV	pontine reticular nucleus, ventral part
5	PoT	posterior thalamic nuclear group, triangular part
6	PP	peripeduncular nucleus
7	PRL	prolactin
8	PSTh	parasubthalamic nucleus
9	PV	paraventricular thalamic nucleus
10	PVA	paraventricular thalamic nucleus, anterior part
11	RCh	retrochiasmatic area
12	RMg	raphe magnus nucleus
13	s5	sensory root of the trigeminal nerve
14	SCh	suprachiasmatic nucleus
15	SFi	septofimbrial nucleus
16	SFO	subfornical organ
17	SG	suprageniculate thalamic nucleus
18	SI	substantia innominata
19	SLEA	sublenticular extended amygdala
20	sm	stria medullaris of the thalamus
21	SNC	substantia nigra, pars compacta
22	SNR	substantia nigra, reticular part
23	SO	supraoptic nucleus
24	SPa	subparaventricular zone of the hypothalamus
25	st	stria terminalis
26	STh	subthalamic nucleus
27	SubCD	subcoeruleus nucleus, dorsal part
28	SubCV	subcoeruleus nucleus, ventral part
29	SuM	supramammillary nucleus

Te	terete hypothalamic nucleus
Tu	olfactory tubercle
VEn	ventral endopiriform nucleus
VLG	ventral lateral geniculate nucleus
VLGMC	ventral lateral geniculate nucleus, magnocellular part
VLGPC	ventral lateral geniculate nucleus, parvicellular part
VLPAG	ventrolateral periaqueductal grey
VLPO	ventrolateral preoptic nucleus
VMH	ventromedial hypothalamic nucleus
VMHc	ventromedial hypothalamic nucleus, central part
VMHdm	ventromedial hypothalamic nucleus, dorsomedial part
VMHvl	ventromedial hypothalamic nucleus, ventrolateral part
VMPO	ventromedial preoptic nucleus
VOLT	vascular organ of the lamina terminalis
VP	ventral pallidum
VRe	ventral reuniens thalamic nucleus
vsc	ventral spinocerebellar tract
VTA	ventral tegmental area
ZI	zona incerta

INTRODUCTION

Prolactin (PRL) is a polypeptide hormone produced at the adenohypophysis, best-known for its role in the development of the mammary gland and milk production [1]. To date, PRL has been attributed more than 300 different biological actions [2] in reproduction, homeostasis, angiogenesis or immunity, among others [3]. To fulfil this array of functions, PRL has different target tissues, including the mammary gland, the uterus and other peripheral organs [3] and, importantly, the brain [4,5]. In the brain, PRL is mainly involved in the regulation of a number of behaviours and physiological processes related to female reproduction and lactation [6] . Indeed, PRL has been described as a pleiotropic hormone responsible for adapting every important aspect of female physiology and behaviour to the demands of motherhood [7].

The key role of prolactin in maternal physiology has traditionally biased neuroendocrinological studies towards its functions in the brain of females, whereas the actions of this hormone in the male brain have drawn less attention. For instance, the distribution of the PRL receptor (PRLR) has been reported for the female [8,9] but not the male rodent brain. One of the main reasons for this bias relates to the fact that male rodent models display low levels of circulating PRL under standard conditions [10], reflecting a limited access and functionality of PRL in the brain. Still, several studies do report acute rises in systemic PRL secretion in males associated to certain physiological and pathological conditions, such as the stress response [11] or sexual behaviour [12].

Regarding the latter, PRL is known to be involved in the control of male copulatory behaviour in rats[13], mice [14], other rodent models [15] and humans[16]. Thus, PRL is released acutely with ejaculation [12] and it is proposed to intervene in the satiation following copulation[17] leading to the refractory period. In fact, chronic hyperprolactinaemia suppresses copulatory behaviour in animal models[18]. In men, hyperprolactinaemia associated to several clinical conditions [19] (including antipsychotic treatments [20]), yields sexual dysfunction and other side-effects [21]. The inhibitory role of PRL on

sexual function is proposed to have an important central component [22,23], but the exact regions and mechanisms through which PRL operates in the male brain are still poorly understood.

In this light, the main aim of this work is to provide a comprehensive description of the distribution of cells sensitive to PRL in the brain of males. To do so, we have supplemented males with a standard dose of exogenous PRL and revealed PRL-responsive cells in the brain by means of the immunohistochemical detection of the phosphorylated form of signal transducer and activator of transcription 5 (pSTAT5). Phosphorylation of STAT5 is a key event in the Jak/STAT signalling cascade of the PRL receptor [3]. In contrast to other methods, e.g. in situ hybridization of PRL receptor (PRLR) mRNA (or its immunodetection), pSTAT5 immunohistochemistry in PRL-supplemented animals reveals those cells that are not just expressing PRL receptors or specific elements of their signalling cascade, but those that are fully responsive to PRL.

The second goal of this work is to compare the pattern of central PRL-derived signalling between male and female mice, in search for intersexual differences. For this purpose, we compare qualitatively and quantitatively the patterns of our PRL-supplemented males with ovariectomised, steroid-primed females treated with an equivalent dose of PRL. Given the key role of PRL in female behaviour and physiology and the broad patterns of PRL-derived signalling in the female mouse brain [4], we hypothesize that the central sensitivity to PRL will show a female-biased sexual dimorphism. In addition, a direct comparison of the distribution of pSTAT5 in the brain of males and females might also be useful for the functional contextualization of PRL input to the male brain.

In accordance with the involvement of PRL in reproductive physiology and behaviour, gonadal steroids are important regulators of PRL function in the brain. In female rodents, estradiol and progesterone have been shown to target most PRL-sensitive neurons in the brain [24] and to regulate PRL signalling at different levels [25–27]. On the other hand, testosterone has been documented to inhibit PRL receptor expression in the pituitary gland of male rats and pituitary PRL release in male rats and mice [28–30]. Beyond these findings, to our knowledge there is currently no available evidence

supporting a regulatory role of testosterone in brain PRL signalling. Hence, in this work we also explore the putative contribution of testosterone in shaping the patterns of PRL responsiveness in the male brain. To this end, we analyse the levels of pSTAT5-ir in the main PRL-responsive regions of the male mouse brain after permanent testosterone withdrawal through castration, as compared to intact male mice, both supplemented with PRL.

By characterizing the patterns of PRL signalling in the male brain and clarifying their dependence on testosterone, we intend to shed light on the roles of PRL in male behaviour and physiology, as well as on the neural substrates for PRL-dependent, male-specific behaviours.

MATERIAL AND METHODS

Animals

For the present study, we used 15 male and 6 female mice of the CD1 strain (Charles River Laboratories, France), aging between 8 and 24 weeks. These animals were housed in polypropylene plastic cages under controlled temperature (24 ± 2 ºC) and lighting conditions (12h:12h; lights ON at 8 am), with *ad libitum* access to food and water. Intact males were housed individually (to avoid inter-male aggression) whereas castrated males and females were group-housed (4 to 6 animals per group, to avoid isolation-derived stress). Animals were treated throughout according to the European Union Council Directive 2010/63/EU (6106/1/10 REV1) and procedures were approved by the Committee of Ethics on Animal Experimentation of the University of Valencia, where the experiments were performed.

Experimental Design

Male mice were randomly assigned to three experimental groups: (1) Male Control (n=3); (2) Male+PRL (n=6) and (3) Castrated+PRL (n=6). Animals of Male Control and Male+PRL groups were left gonadally intact, whereas males from Castrated+PRL group underwent orchidectomy. Following

the procedure by Brown and collaborators[5,31], Male+PRL and Castrated+PRL groups received an exogenous PRL supplementation 45 minutes prior to perfusion.

In order to compare central PRL sensitivity between males and females, we also included in our study a group of ovariectomised, steroid-primed and PRL-supplemented females, which belonged to a sample of a previously published set of experiments [4]. The steroid-replacement schedule conducted on these females intended to emulate the proestrus-estrus phase of the estrous cycle [32]. Given the considerable variability of pSTAT5-ir in the brain of freely-cycling females, likely due to gonadal steroid influence on PRL signalling [4], this group provided a more stable comparison than freely-cycling females and a more accurate comparison than estrous cycle screening.

Data from these four groups of animals (Male Control; Male+PRL; Castrated+PRL and Female+PRL) allowed us to: a) characterize the pattern of PRL-derived signalling in the male brain by analysing the distribution of pSTAT5-ir in group Male+PRL; b) analyse the effect of PRL supplementation on pSTAT5-ir distribution by comparing the Male Control with Male+PRL groups; c) explore sexual dimorphism in the central sensitivity for PRL by comparing Male+PRL with Female+PRL groups; and finally, d) assess the modulatory role of testosterone on PRL signalling in the male brain through the comparison of the Male+PRL and the Castrated+PRL groups.

Ovariectomy and Orchidectomy

Males of Castrated+PRL group were orchidectomised at approximately 12 weeks of age. Briefly, animals were deeply anaesthesised with i.p. ketamine (Imalgene 500, Merial, Toulouse, France, 75mg/kg) and medetomidine (Domtor 1mg/ml, Esteve, Barcelona, Spain, 1 mg/kg) and surgery was performed via a single midline incision on the scrotal sac. After surgery, i.p. atipamezol hydrochloride (Antisedan, Pfizer, New York, USA, 1 mg/kg) was administered to reverse anaesthesia and s.c. butorphanol tartrate 1% (Torbugesic, Pfizer, New York, USA, 20 µl) was regularly administered through the postsurgical period for pain control. Castrated males had 2-3 weeks of recovery under undisturbed housing conditions before the beginning of the experiments.

Experimental females underwent ovariectomy 9-10 weeks after birth under identical pharmacological conditions as explained for males [4]. Surgery was conducted through two incisions on both sides of the back of the animal. Ovariectomized females had at least seven days of recovery after surgery.

Hormone Treatments

Experimental females underwent a steroid replacement schedule [4] consisting of a sustained treatment with 17-β estradiol and an acute treatment with progesterone. In accordance with the experimental induction of estrus [32], estradiol was administered on a slow-release profile during 7 days, by means of the subcutaneous placement of silastic tubing implants (Dow Corning Corporation, Midland, MI, USA) filled with 20 µg/ml β-estradiol (Sigma, St Louis, MO, USA) diluted in sunflower oil. Silastic tubing had an inner diameter of 1.67 mm and an outer diameter of 2.41 mm, and implants were cut to a length of 20 mm. Implants were inserted subcutaneously on the lumbar region under isoflurane anaesthesia (Isoflo, Esteve Veterinaria, Barcelona, Spain). Animals were also administered a subcutaneous dose of 20 µl butorphanol tartrate 1% (Torbugesic, Pfizer, New York) during implant placement surgery, for pain control. By contrast, progesterone (Sigma, St Louis, MO, USA) was administered to females acutely in a 500 µg subcutaneous injection, diluted in sunflower oil, in the morning of the seventh day of estradiol treatment, specifically 3 hours and 45 minutes prior to perfusion. In order to make males and females directly comparable for the analysis of sexual dimorphism, males of the Male+PRL group received vehicle treatment parallel to that of females (implants with sunflower oil, plus s.c. oil injection 3h 45 minutes prior to perfusion).

Regarding PRL supplementation, except for the Male Control group (which received vehicle injection), all experimental groups were administered an acute 5 mg/kg i.p. dose of ovine PRL (Sigma, St Louis, MO, USA) 45 minutes before perfusion. The dose and timing of PRL administration rendered homogenous, supraphysiological circulating levels of PRL and ensured that the peak of STAT5 phosphorylation in response to a PRL challenge coincided with perfusion [5]. This is intended to

reveal the maximal potential response patterns among experimental animals and also to allow direct comparison between experimental groups, regardless of sex (male vs female) or physiological condition (intact vs castrated males).

Tissue Collection and Histological Processing

Animals received an overdose of sodium pentobarbital (Vetoquinol, Madrid, Spain) and were transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. Brains were carefully extracted and post-fixed overnight through immersion in the same fixative, then cryoprotected by immersion in 30% sucrose in 0.01 M PB until they sank (2-3 days). Using a freezing microtome (Microm HM-450, Walldorf, Germany) brains were sectioned in five parallel series of 40 µm thick coronal sections. Series were stored in PB-30% sucrose at -20ºC until their use.

Immunohistochemistry for pSTAT5

Immunohistochemistry was conducted in free-floating sections under light shaking at room temperature (25ºC) unless otherwise stated. Immunohistochemistry protocol was adapted from Brown et al.[5,31]. Tissue sections were thoroughly rinsed between stages for at least three 10-min washes in TRIS-buffered saline, 0.05M, pH 7.6 (TBS). After thawing, sections underwent an initial antigen retrieval step, consisting in two sequential 6-minute incubations in 0.01 M TRIS buffer (TB), pH 10 at 85ºC, and brought quickly to room temperature in between. Tissue was then incubated in: a) 1% hydrogen peroxide (H_2O_2) for 30 minutes, for endogenous peroxidase inhibition; b) 2% BSA, 2% goat serum and 0.3% Triton X-100 in TBS for 1h, in order to block unspecific labelling; c) rabbit monoclonal anti-pSTAT5 primary antibody (Phospho-Stat5 Tyr694 (D47E7), Catalog number #4322, Cell Signaling Technology, Danvers, MA) diluted 1:500 in TBS plus Triton X-100 0.1% for 72 h at 4ºC; d) biotinylated goat anti-rabbit IgG (Vector Laboratories, Peterborough, UK; RRID AB_2313606) 1:200 in TBS for 90 minutes; and e) avidin-biotin-peroxidase complex (ABC Elite kit; Vector Laboratories; RRID AB_2336819) in TBS for 90 minutes. Peroxidase label was developed using 0.005% 3-3'-diaminobenzidine (Sigma) and 0.01% H_2O_2 in TB pH 7.6 for about 15 minutes, obtaining

thereby a brown nuclear staining. Sections were rinsed in TB and mounted onto gelatinized slides, dehydrated in graded ethanol, cleared with xylene and coverslipped with Entellan.

The specificity of the anti-pSTAT5 primary antibody was demonstrated by means of an antibody competition assay (Supplementary Figure 1).

Analysis of pSTAT5 Immunoreactivity

The patterns of pSTAT5-ir distribution were mapped for the brain of intact males (Male+PRL) and females (Female+PRL) and illustrated semiquantitatively in camera lucida drawings of selected brain sections (Fig. 1). In addition, a quantitative assessment of the density of cells showing pSTAT5 immunoreactivity (pSTAT5-ir) in representative brain sites was performed for PRL-treated females and males, both intact and castrated. To do so, we first selected frames of the chosen nuclei using the stereotaxic atlas of Paxinos and Franklin (2004). Then, we obtained photomicrographs of these frames in both hemispheres using a digital camera (Leica DFC495) attached to a microscope Leitz DMRB (Leica AG, Germany). Thereafter, images were processed and analysed using ImageJ. Briefly, we subtracted background light and converted the RGB colour image to greyscale by selecting the green channel. Then, we binarised the greyscale image setting the 75% of the mode of the histogram as a threshold, thus including every pixel below this threshold as positively labelled. Using the ImageJ commands “fill holes”, “open” (3 iterations) and “watershed”, we filtered small particles (noise or fragmented nuclei) and separated fused objects. The resulting particles were additionally filtered by area (larger than 70 μm^2, corresponding to an approximate diameter of 9.4 µm) and finally counted automatically. We calculated the mean (interhemispheric) density of pSTAT5-imunoreactive cell nuclei for each specimen by dividing the counts for both hemispheres by the area of both frames.

Statistical Analysis

We performed statistical analysis of the resulting data on the SPSS software package. After checking for normality (Kolmogorov-Smirnov test with Lilliefors' correction) and homogeneity of variances (Levene's test), we conducted three different statistical comparisons. First, we compared levels of pSTAT5-ir density between intact males (Males + PRL) and ovariectomised, steroid-primed females (Female+PRL), in search for intersexual differences. Then, we checked for the effect of orchidectomy on male pSTAT5-ir levels by comparing intact (Male+PRL) and castrated (Castrated+PRL) males. For both analyses, the samples fulfilling the criteria for a parametric analysis were subject to an independent t-test, whereas the samples of the remaining nuclei were subject to a non-parametric Mann-Whitney test. For each statistical test, we applied a significance level of 0.05.

RESULTS

The main focus of this section is to characterise the central patterns of pSTAT5-ir found in male mice in the presence of high levels of circulating PRL (Male+PRL group), as an indicator of PRL-derived signalling occurring in the male brain. In the same vein, we compare the distribution of pSTAT5-ir between PRL-supplemented males (Male+PRL group) and non-supplemented males (Male Control group). Then, we analyse intersexual differences in PRL-derived signalling in the mouse brain. For this purpose, we compare the patterns of pSTAT5-ir in the male (Male+PRL group) and female (Female+PRL group) brains and assess the density of pSTAT5-ir in a number of brain regions displaying PRL-derived signalling in both males and females. Finally, we explore the effect of testosterone withdrawal in the PRL-derived signalling of the male brain by comparing pSTAT5-ir levels between intact and castrated male mice. For the following anatomical descriptions, we adhere to the neuroanatomical terminology proposed by Paxinos and Franklin[33] (see also list of abbreviations above).

A. Patterns of pSTAT5 immunoreactivity in the male mouse brain: effect of PRL supplementation

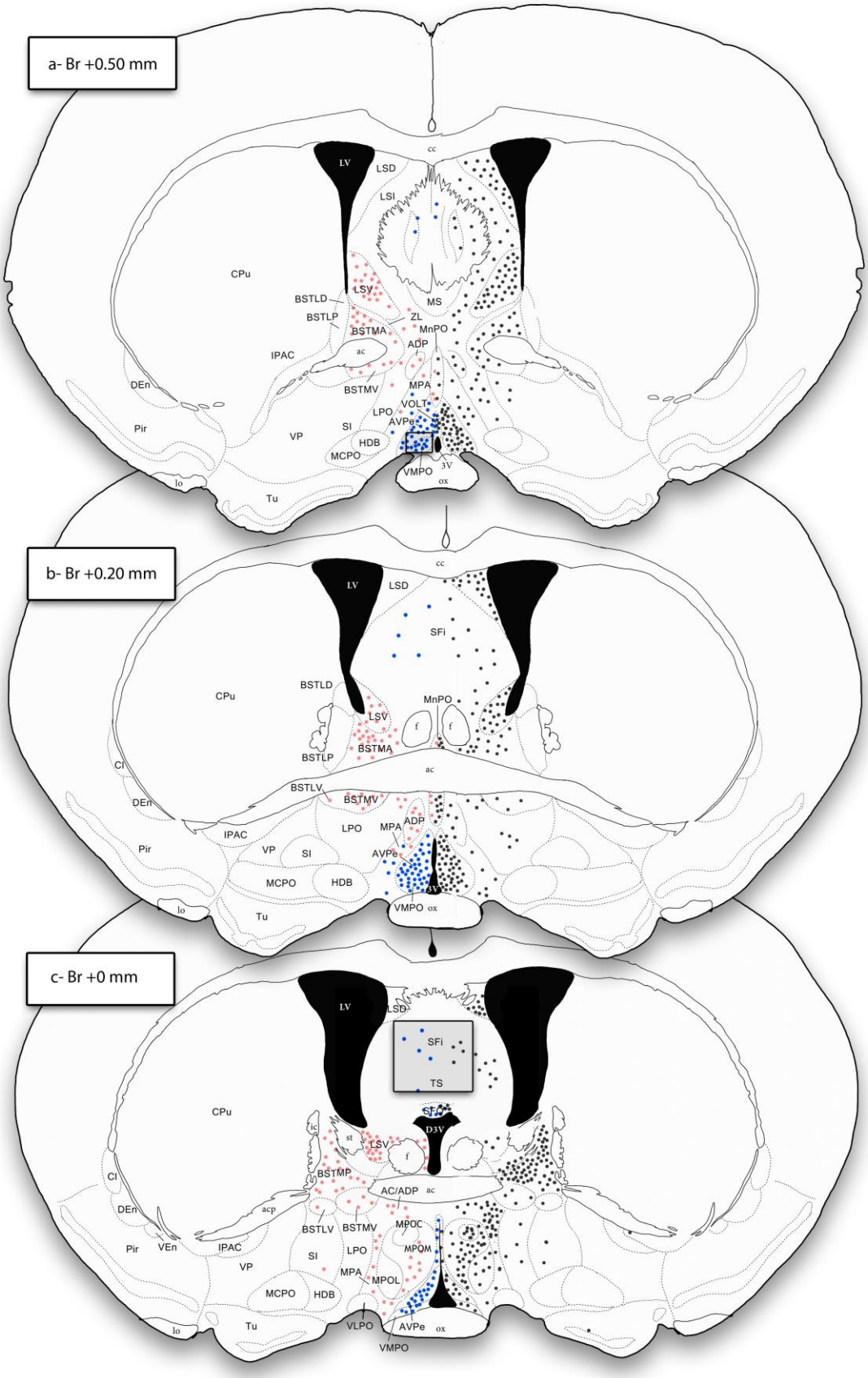
Immunohistochemistry for pSTAT5 produced a defined staining in the examined brain tissue, which was restricted to the cell nucleus (see Fig. 2), as shown in our previous work [4]. Labelling was

suppressed by pre-incubation of the antibody with the phosphorylated peptide used for immunization, but not when the antibody was incubated with the equivalent, non-phosphorylated peptide (see Suppl. Fig. 1). Therefore, we safely assume that, in our immunohistochemical procedure, the antibody is detecting specifically pSTAT5.

Figure 1 maps, on the left side of the brain, the distribution of pSTAT5-ir observed in PRL-supplemented males (blue dots; for the significance of red dots see below). On the right side of the brain, black dots show the distribution of cells expressing PRL receptor (PRLR) in the male mouse brain, as reported by the Allen Brain Institute (see Suppl. Fig. 2). Figure 2 shows photomicrographs illustrating pSTAT5-ir in selected brain nuclei for all of our experimental groups.

Males supplemented with PRL (Male+PRL) showed moderate-to-dense pSTAT5-ir in several nuclei of the hypothalamus and cerebral hemispheres (Fig. 2, left column), as well as in the choroid plexus (not shown). In the telencephalon, pSTAT5-ir was mainly observed in the septum, specifically in the area comprising the septofimbrial nucleus (SFi) and the triangular septal nucleus (TS) (Figs. 1c and 1b). In this region, the subfornical organ (SFO) also showed some immunoreactive cells. In addition, the posterodorsal nucleus of the medial amygdala (MePD) displayed scarce pSTAT5-ir, with few immunolabelled cells restricted to the upper corner of the nucleus, in close contact to the intramygdaloid BST (BSTia, see Figs. 1f and 2f). Importantly, all these brain nuclei display PRLR mRNA expression in the male mouse (Fig. 2 right side).

In the hypothalamus, pSTAT5-ir was present in the preoptic, anterior and tuberal regions. In the preoptic hypothalamus (Figs. 1a-d and 2j), pSTAT5-ir cells were concentrated in the juxtaventricular nuclei, namely the vascular organ of the *lamina terminalis* (VOLT) and the anteroventral periventricular (AVPe), the ventromedial preoptic (VMPO) and the Pe nuclei. In addition, the adjoining MPA displayed a few immunolabelled cell nuclei.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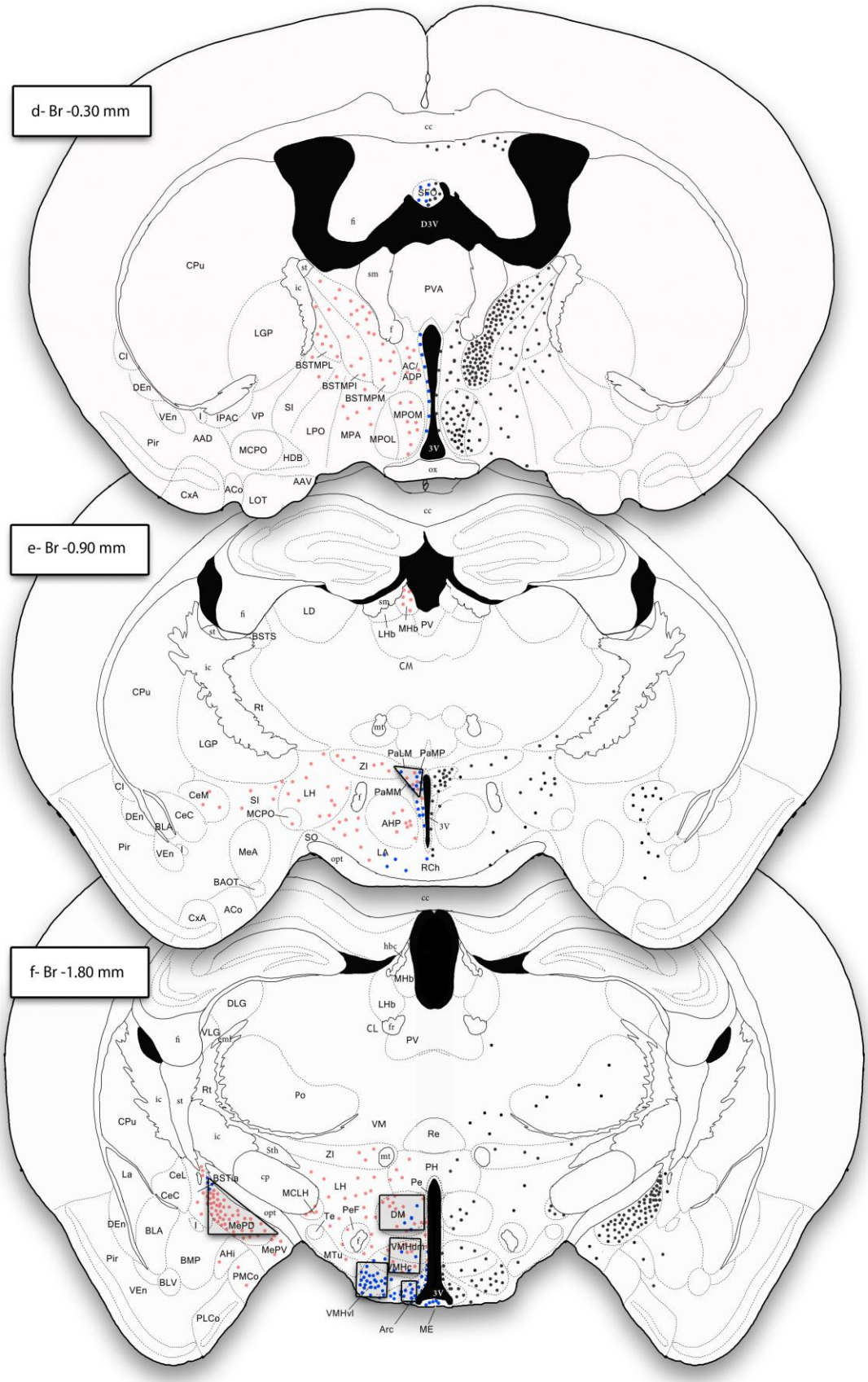
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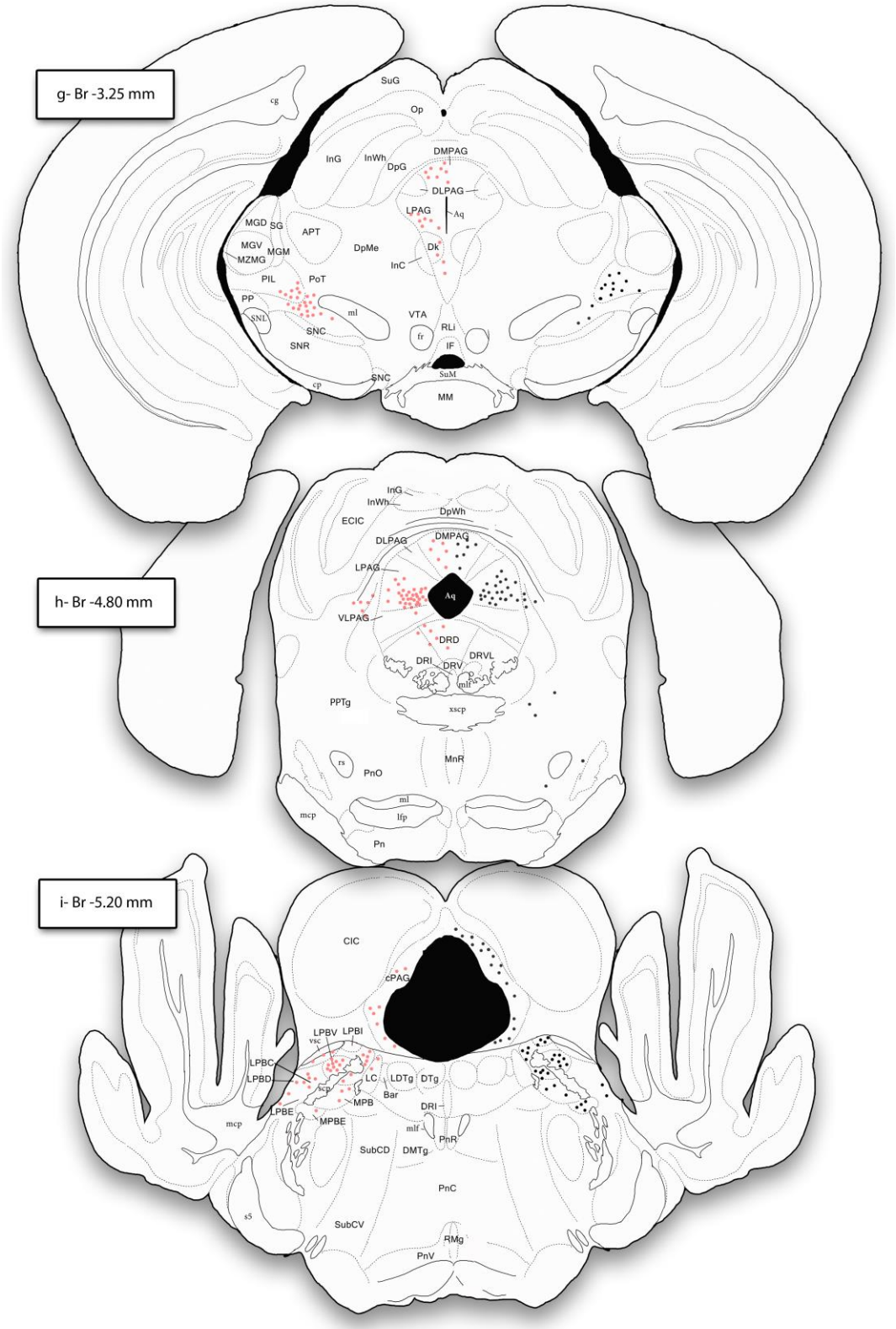

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2 **Figure 1. Mapping of pSTAT5 immunoreactivity and prolactin receptor expression in the brains of**

3 **male and female mice**

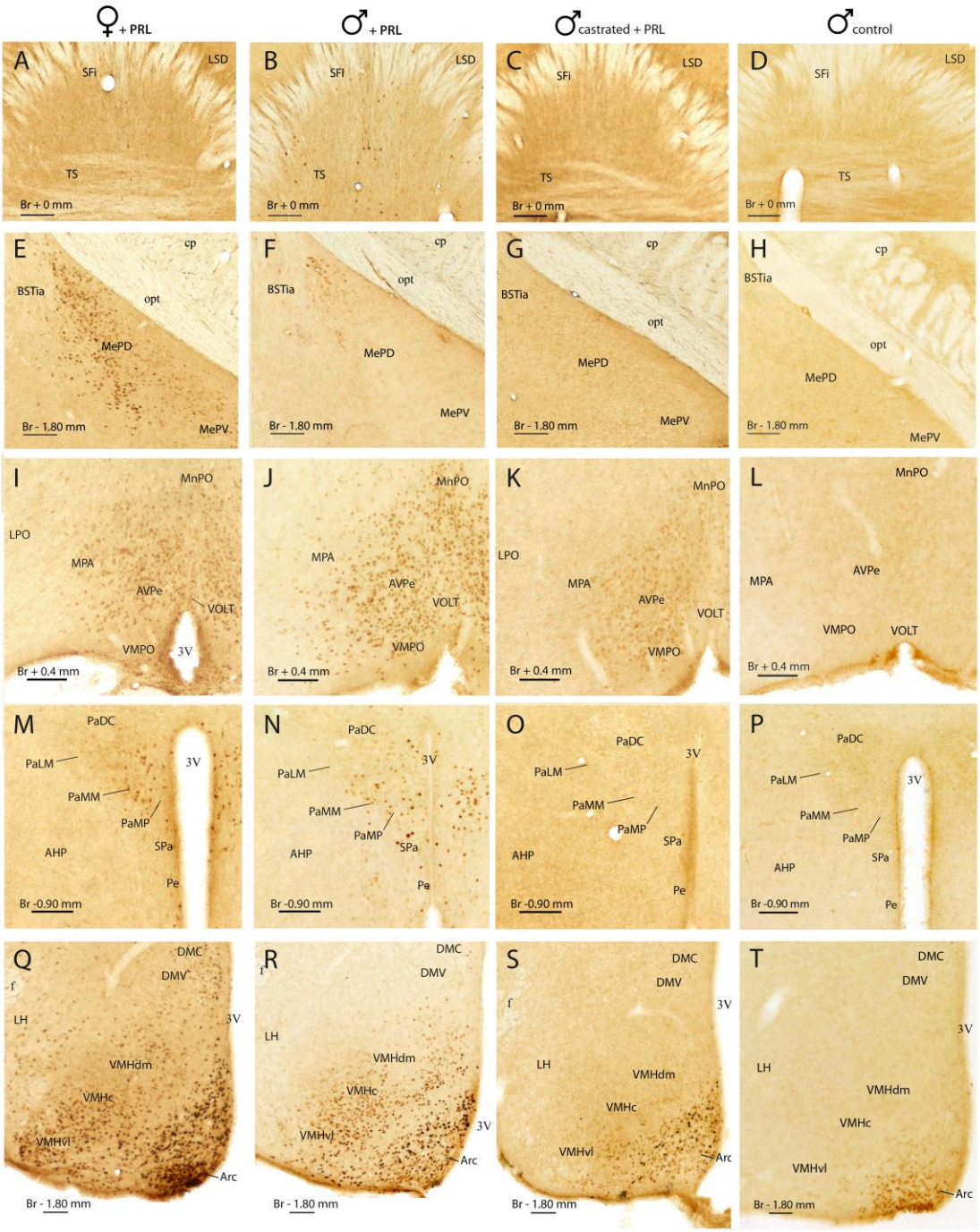

Figure 2. Representative examples of pSTAT5 immunoreactivity in the brain of female, intact male and castrated male mice

In the anterior hypothalamus, pSTAT5-ir was once again restricted to juxtaventricular structures, the paraventricular nucleus (Pa) and the Pe (Figs. 1e and 2n), as well as the retrochiasmatic area (RCh, Fig. 1e). In the Pa, labelling was restricted to the medial aspect of the nucleus, mainly to the ventral

(PaV), medial magnocellular (PaMM) and medial parvocellular (PaMP) subdivisions, with very few labelling observed in the lateral aspect of the nucleus (PaLM) or the subparaventricular nucleus (SPa). In the tuberal hypothalamus (Figs. 1f and 2r), the arcuate nucleus (Arc) displayed abundant pSTAT5-ir, with some immunolabelled cells displaced into the median eminence (ME). In some males, the dorsomedial nucleus (DM) displayed few scattered immunolabelled cells. Finally, pSTAT5-ir appeared in the ventromedial hypothalamic nucleus (VMH), with higher levels in the ventrolateral subdivision (VMHvl) and sparse labelling in the rest (VMHc and VMHdm).

In contrast to the Male+PRL group, non-supplemented males (Male Control, Fig. 2, rightmost column), were virtually devoid of pSTAT5-ir, except for some labelled cells in the ventral aspect of the arcuate nucleus (Arc, Fig. 2t) and the anterior aspect of the periventricular nucleus (Pe, not shown).

B. Comparative analysis of pSTAT5-ir the male and female mouse brain

In comparison to males, ovariectomised, steroid-primed females supplemented with PRL (Female+PRL) showed a more extensive distribution of pSTAT5-ir (Fig. 1, Fig. 2 middle-right column), which matches previous descriptions conducted on freely-cycling female mice[4]. To summarize, labelling in our experimental females was widespread in the basal telencephalon and hypothalamus, but was also found within thalamic, midbrain and brainstem structures. To allow a direct comparison between sexes, Figure 2 shows the distribution of pSTAT5-ir in equivalent brain sections of male and female mice. In addition, in the semischematic drawings of brain sections depicted in Fig. 1, pSTAT5-ir labelling that was common to males and females is shown as blue spots, whereas labelling exclusively present in females is illustrated as red spots. Importantly, there was not a single brain site where labelling was exclusively found in males. In other words, after administration of equivalent doses of exogenous PRL, males show pSTAT5-ir in a reduced number of brain centres, whereas females show immunoreactive cells in the same nuclei, plus a set of additional brain centres. This represents a clear case of sexual dimorphism in favour of females.

We also checked for quantitative sexual dimorphism in PRL-derived signalling, by analysing the density of pSTAT5-ir in the main nuclei showing pSTAT5-ir in PRL-supplemented intact males and steroid-primed ovariectomised females. We designed counting frames for the following brain nuclei: the AVPe/VMPO region, the Pa, the Arc, the DM, the VMHvl, the area comprising the central and dorsomedial VMH (VMHc and VMHdm, respectively), the MePD and the SFi (Fig. 1). Using ImageJ we assessed the density of pSTAT5-ir in each nucleus (mean hemispheric cell density) and performed separate statistical tests on each of the analysed nuclei to compare Male+PRL and Female+PRL groups. When data fulfilled the criteria for a parametric statistical analysis (the AVPe, the Arc, the Pa and the VMHvl), we performed t-tests for independent samples. For the remaining nuclei (the DM, the VMHc, the MePD and the SFi), we performed a non-parametric Mann-Whitney test. Figure 3 shows the results of these analyses. Females showed a general trend towards higher levels of pSTAT5-ir than males. This effect reached statistical significance in the Arc (t(10)=3.043, p=0.012), the DM (U=32.0; p=0.026) and the MePD (U=30.0; p=0.004), although the AVPe/VMPO also showed an almost significant trend towards higher pSTAT5-ir density in females (t(9)=2.183; p=0.057).

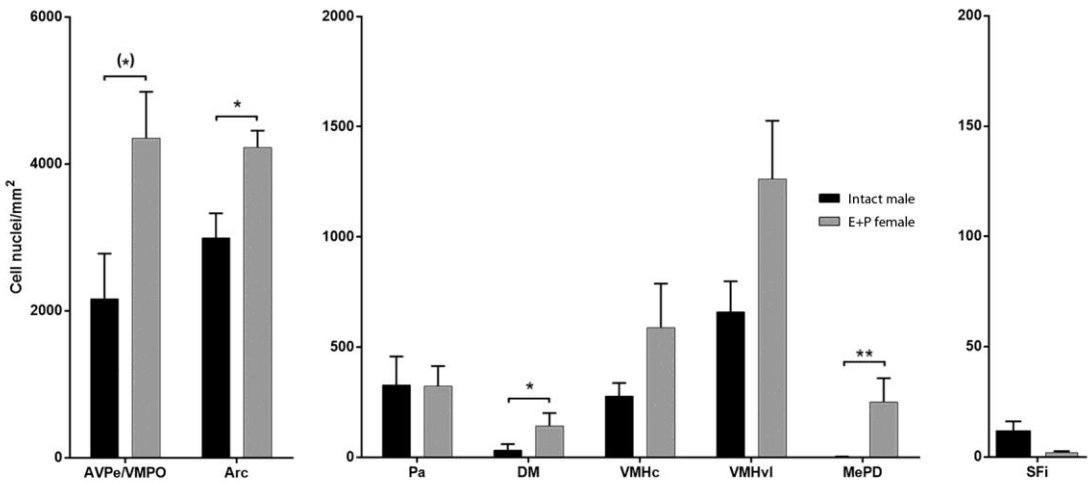

Figure 3. Quantitative analysis of pSTAT5 immunoreactivity in selected brain regions of female and male mice

C. Effect of testosterone withdrawal in PRL-derived signalling in the male mouse brain

Finally, we explored the putative role of testosterone in the regulation of PRL-derived signalling in the male brain. To do so, we compared pSTAT5-ir patterns between intact, PRL-supplemented males (Male+PRL; Fig. 2, leftmost column) and a sample of castrated, PRL-supplemented males (Castrated+PRL; Fig. 2, rightmost column). In qualitative terms, castrated and intact male mice displayed similar patterns of pSTAT5-ir, but castrated males showed apparently lower levels of pSTAT5-ir. Quantitative analysis of the labelling in both groups (using the same methodology and exploring the same subset of nuclei analysed in the previous section) allowed comparing pSTAT5-ir between gonadally intact and castrated males by means of independent t-tests for the Arc and AVPe and non-parametric Mann-Whitney tests for the rest of the analysed regions. The results of this statistical comparison reveal a significant effect of testosterone withdrawal in diminishing the levels of pSTAT5-ir in the Pa (U= 33.00; p=0.004), in both the ventrolateral and central/dorsomedial VMH (U= 30.00, p=0.015 for both frames) and in the SFi (U= 26.00, p=0. 05). In the remaining analysed nuclei, the density of pSTAT5-ir cells was similar in Male+PRL and Castrated+PRL animals, as no statistical differences were found (p>0.1) (Fig. 4).

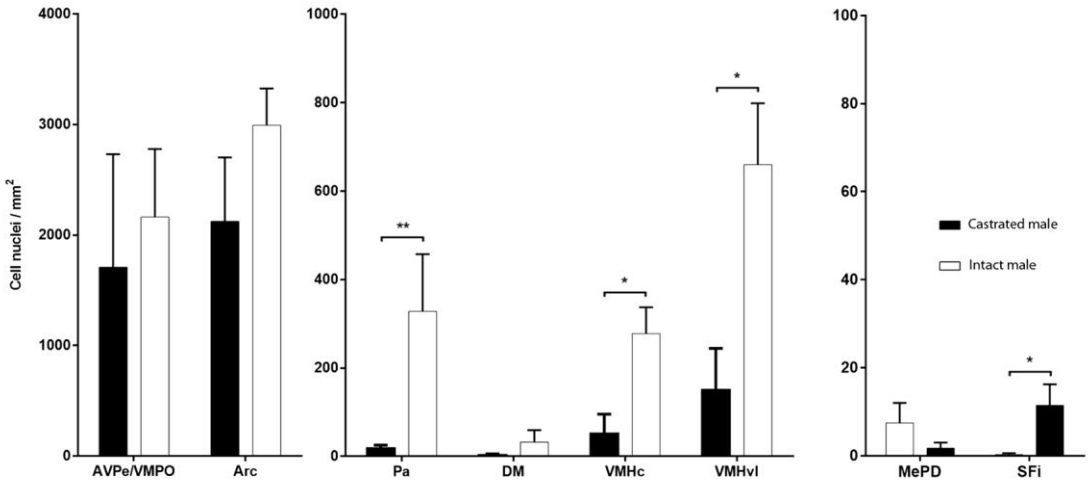

Figure 4. Effect of testosterone withdrawal in pSTAT5 immunoreactivity of the male mouse brain

DISCUSSION

The present study examined, for the first time, the responsiveness of the brain of males to PRL. Specifically, we analysed the distribution and steroid regulation of PRL-derived signalling in the male mouse brain, by means of the immunohistochemical detection of pSTAT5. We also compared the pattern of pSTAT5 immunoreactivity in the brain of males and females, aiming at assessing its possible sexual dimorphism. In this section, we will first analyse the distribution of pSTAT5 in the brain of male mice, with or without exogenous PRL administration, and compare it to the pattern of expression of the PRLR, as shown in in situ hybridisation material provided by the Allen Brain Institute (see Figure 1 and supplementary Figure 2). Then, we will comment on the observed sexually dimorphic differences in central sensitivity to PRL. Next, we will discuss the role of testosterone as a putative regulator of central PRL signalling and on the mutual regulatory relationship of PRL and testosterone. We will conclude by reviewing how our findings contribute to elucidate the neuroanatomical substrate of some putative actions of PRL in the brain of males.

Prolactin signalling in the male mouse brain, PRL supplementation and PRL receptor expression

Although the actions of PRL in the female mouse brain have received considerable attention due to the role of PRL in female reproduction and motherhood[34], this work is, to our knowledge, the first descriptive analysis of the distribution of PRL-derived signalling in the male mouse brain. In PRL-supplemented male mice (Male+PRL group), brain regions positively labelled for pSTAT5-ir were located mainly in the hypothalamus (the AVPe and VMPO, MPA, Pe, Pa, Arc, DM and VMH) and, to a lesser extent, in the telencephalon (SFi in the septum and MePD in the amygdala), with no additional pSTAT5-ir detected in any of the remaining brain divisions (Fig. 1).

Importantly, this pattern of pSTAT5-ir was highly dependent on systemic supplementation with high doses of exogenous PRL, whereas non-supplemented controls (Male Control group) were virtually devoid of pSTAT5-ir, except for some faintly labelled cells in the ventral Arc (Fig. 2t) and in the anterior periventricular hypothalamus (not shown). These results suggest, in the first place, that the

patterns of pSTAT5-ir we report in this work are specifically produced by PRL, whereas other endocrine agents signalling through the Jak/STAT pathway, such as growth hormone[35], do not contribute substantially to STAT5 phosphorylation in the studied cases. This is further supported by the fact that pSTAT5 appears only in centres that show expression of PRLR in the brain of male mice (Figure 1; Suppl. Fig. 2), whereas the distribution of growth hormone receptor does not fit the pattern of pSTAT5-ir[35,36].

In addition, the reduced pSTAT5-ir found in males not supplemented with exogenous PRL indicates that, in basal conditions, PRL signalling in the male brain is very low, what fits the circulating levels reported for the hormone for males[10,37]. In fact, the presumed location of the TIDA neurons (as revealed by tyrosine hydroxylase immunohistochemistry), the dorsal aspect of the Arc[38], displays scarce pSTAT5-ir in Male Control mice (Fig. 2t). This supports the view of very low circulating PRL levels in male mice, at least in the light period of the day [37]. In line with these observations, PRL would influence the brain of males especially under some physiological conditions in which there is substantially increased hypophyseal or extra-hypophyseal PRL release, for instance after mating[39] or as part of the stress response [40].

Sexually dimorphic PRL-derived signalling in the mouse brain

In comparison to males, female mice displayed a more extensive distribution of pSTAT5-ir, mainly in the hypothalamus and basal telencephalon, but also present in nuclei of the thalamus, midbrain and brainstem. Patterns of pSTAT5-ir in males were always a completely overlapping fraction of those of female mice. In other words, we found no single brain structure labelled in males but not in females. Furthermore, a number of nuclei with pSTAT5 expression in both sexes displayed significantly higher pSTAT5-ir density in females than in males (the AVPe/VMPO, Arc, DM and MePD), but none of the analysed nuclei showed higher levels of pSTAT5-ir in males (Fig. 3). Altogether, our study reveals a clear, female-biased sexual dimorphism in PRL-derived signalling in the mouse brain upon exogenous PRL administration. In fact, this sexually dimorphic responsiveness to PRL would be extensive to

specific functional systems of the brain, for instance to the Sociosexual Behavioural Network (SBN), the core system for the integration of social and reproductive behaviour[45]. In contrast to females, several nodes of the SBN (the lateral septum, BST, medial amygdala or PAG, see Figs. 1 and 2) display no pSTAT5-ir or marginal levesl (MePD) in males, even after exogenous PRL administration, although they do express the PRL receptor (Fig. 1 and Supplementary Fig. 2). This suggests that, in these areas, either PRL responsiveness is regulated through different receptor activation pathways in males and females, or PRL signalling is downregulated in males, leading to a lower implication of PRL in the regulation of sociosexual behaviour in males. However, this does not exclude a role in some male-specific behaviours, as we will further discuss below.

Prolactin and Testosterone: Mutual Neuroendocrine Regulation

One of the main outcomes of the present work is the identification of a putative regulatory role of testosterone in PRL signalling in the male mouse brain. Orchidectomy and consequent testosterone withdrawal led to a significant decrease in pSTAT5-ir density in the SFi, the Pa and both the central/dorsomedial and lateral VMH (Fig. 4). This indicates a positive regulatory role of testosterone on PRL signalling in those brain regions. The actual mechanism of this process is not clear. Even though testosterone has documented inhibitory effects on hypophyseal PRL release in male rats[29] and mice[30], the effect of castration on pSTAT5-ir cannot be attributed to differences in circulating PRL, since intact and castrated males were supplemented with equivalent levels of exogenous PRL. In addition, this effect is not likely explained by a facilitated access of systemic PRL to the brain, as our results reveal that the positive effect of castration on pSTAT5-ir is not generalised but rather specific and restricted to discrete brain sites. Therefore, our results suggest that testosterone might upregulate PRLR expression or perhaps signalling downstream the PRLR in specific neural populations. Further research is required to elucidate the specific action of testosterone on PRL signalling in male mice.

An additional question of interest is the identity of the actual biological modulator of PRL signalling we report here. Testosterone can be locally metabolized into two different neuroactive steroids: it can be reduced to dihydrotestosterone[42], which binds to androgen receptors, or aromatised to estradiol[43] that then binds to oestrogen receptors. Our results make it very unlikely that this regulation occurs through the aromatisation of testosterone to estradiol. Although all the nuclei where PRL signalling is affected by castration express at least one form of oestrogen receptor[44], none of them displays aromatase activity in the male mouse brain[45]. Conversely, all of the nuclei where PRL-derived signalling decreased with castration express androgen receptors, with the Pa and VMH showing very high levels[45]. Hence, evidence suggests that the effect of testosterone on PRL signalling that we report here is likely mediated by androgen rather than oestrogen receptors.

In addition to the reported regulation of testosterone on central PRL signalling, our data, together with published evidence, suggest a reciprocal regulatory action of PRL on the hypothalamus-pituitary-gonadal (HPG) axis and ultimately on testosterone release. According to the literature, PRL appears to upregulate the HPG axis and testosterone secretion in males. For instance, Bartke and collaborators[14] reported increases in FSH and LH in hyperprolactinaemic male mice, and Chandrashekar and Bartke[46] observed that exogenous PRL infusions stimulate the increase in serum testosterone in male rats, whereas immunological disruption of endogenous prolactin eliminated this effect. This regulatory action of PRL on the HPG axis likely occurs through the kisspeptin system. Kisspeptin plays an essential role in reproduction, as it regulates gonadotropin-releasing hormone (GnRH) expressing neurons in the brain[47], and thereby activate the HPG axis. Therefore, the expression of PRL receptors by kisspeptin neurons[48] allows for a direct role of PRL on HPG axis control and testosterone secretion. In this context, our results provide support for the hypothesis that the brain kisspeptin system is subject to PRL regulation in male mice, too, since our sample of male mice displayed substantial pSTAT5-ir in the hypothalamic nuclei containing kisspeptin-positive neurons[49], the AVPe and the Arc (Figs. 2j and 2r). Nevertheless, this possibility requires experimental

proof by assessing the coexpression of kisspeptin and pSTAT5 immunoreactivity in the aforementioned nuclei.

Altogether, our results and previously published evidence in mice and rats suggest that PRL and testosterone have complex and reciprocal regulatory actions. On the one hand, testosterone inhibits hypophyseal PRL release in male mice[30], but it also appears to enhance PRL signalling in some brain nuclei (Fig. 4). On the other hand, PRL might act onto hypothalamic kisspeptin neurons to stimulate the release of testosterone by the testes.

Prolactin signalling in the male brain: functional implications

Despite the reported female bias in the central responsiveness to PRL, our findings confirm that the male mouse brain is indeed responsive to PRL, implying that PRL likely exerts certain actions in the male mouse brain, too. The nature of these actions is mostly unknown, but PRL signalling is virtually absent in the nodes of the socio-sexual brain network (SBN; with the exception of the VMH), thus suggesting that PRL has a minor direct role in regulation of social behaviours (sexual, agonistic, affiliative and/or parental), although a putative indirect role cannot be excluded either (for instance through the regulation of central oxytocinergic pathways, see below). When comparing males with females, PRL action in the SBN shows a clear female-biased dimorphism (see above) where it certainly regulates sociosexual (including maternal) behaviours. Indeed, the differential action of hormones in males and females is a defining feature of the SBN [41] that explains the gender-specific endocrine regulation of most social behaviours[4,24].

In this respect, the most prominent (but poorly understood) function of PRL in males is the regulation of sexual behaviour [3,16]. It has been shown that central action of PRL after mating suppresses sexual behaviour as part of the satiatory mechanisms operating after ejaculation [17,22]. Similar inhibitory action of PRL onto sexual behaviour occurs under chronic hyperprolactinaemia[50–52]. Prolactin is proposed to do so by modulating the function of some of the major dopaminergic circuits in the brain, closely involved in male sexual function[53]. This hypothesis is supported by

experimental evidence that acute elevations of circulating PRL after copulation lead to significant decreases in dopaminergic activity in the target sites of dopaminergic pathways, including the striatum [23,54] and the MPA region [55]. It is suggested that this decreased dopaminergic activity would occur by PRL action onto cells giving rise to ascending dopaminergic pathways, e.g. the incerto-hypothalamic, mesocorticolimbic and nigrostriatal projections[17]. However, according to our findings, virtually none of the nuclei originating these dopaminergic pathways displays PRL-derived signalling in male mice, even after injection of exogenous PRL (Fig. 2), neither PRLR expression (Fig. 1 and supplementary Fig.2). In sum, our results suggest that the proposed action of PRL over these dopaminergic systems should rather be indirect, through nuclei afferent to the dopaminergic cells.

In this context, the VMH is closely related to sexual behaviour, it is sexually dimorphic[56], known to promote the expression of copulatory behaviour in females[57] and to inhibit mounting in male rats [58]. According to our results, the VMH is substantially responsive to PRL in males, showing in fact similar levels of pSTAT5-ir as in females (Figs. 2q, 2r and 3). In addition, our data on castrated male mice suggest that this responsiveness is also dependent on testosterone (Fig. 4). Hence, PRL could be able to regulate male sexual behaviour also through the VMH in an androgen-dependent fashion.

Related with this, research conducted fundamentally in male rats has established that the magno- and parvocellular oxytocin (OXT) cells in the Pa are involved both in the peripheral and central regulation of male sexual behaviour. On the one hand, peripheral OXT released from Pa cells [59,60] facilitates genital reflexes, erection and copulatory behaviour in response to sexual cues and sexual interaction [61]. On the other hand, OXT also regulates sexual behaviour through its central action, affecting key neural centres for sexual behaviour, such as the MPO [62–64] and VMH (see above). As described before, our findings reveal substantial levels of pSTAT5-ir in the Pa of male mice, comprising the magno- and especially the parvocellular division of the nucleus. This responsiveness to PRL is also dependent on testosterone, since castrated males display significantly lower levels of pSTAT5-ir in this nucleus (Fig. 3). Summarizing, PRL could regulate copulatory behaviour expression

through its testosterone-dependent action over two specific hypothalamic nuclei, the Pa (specifically through the OXTergic populations of this nucleus) and the VMH.

The interaction between OXT and PRL at the level of the Pa can also have a role in the regulation of parental behaviours. There is solid evidence indicating that PRL [65] and OXT are fundamental in the control of maternal behaviour [66,67] and it is likely that they have an equivalent role in males. Tachikawa and collaborators[68] showed that paternal behaviour can be induced in male mice (which are usually infanticidal) by postcopulatory cohabitation with a maternal female. This behavioural induction of paternal behaviour is dependent on OXT release in the nucleus accumbens (Acb), as it is disrupted in knock out male mice for CD38 (a second messenger involved in the release of OXT)[69] and rescued subsequently after the infusion of OXT in the Acb [70]. Dolen and collaborators [71] demonstrated that the OXT innervation of the Acb arises in the Pa (see also[72]). Hence, the input to the Acb from OXTergic Pa cells appears to be instrumental in the induction of paternal care in male mice. Our results, in turn, suggest that OXT cells in the Pa are probably responsive to PRL in male as well as female mice (Figs. 2m and 2n). Therefore, PRL might be involved in the induction of parental behaviour in male mice, too. In support of this hypothesis, PRL facilitates the pup-sensitisation process in male rats [73] in a similar fashion as in females [65] and has further roles in other experimental models for paternal behaviour [74].

Exogenous PRL in male rats has also been recently shown to have pro-social effects, in particular in social recognition and social learning paradigms in which OXT has also been involved [75]. The OXT neurons in the Pa, probably sensitive to PRL according to our results, may mediate the PRL effects on OXT-dependent social behaviours[71].

Our results indicate a modulatory role of PRL onto cells of the Arc and Pe nuclei of males, which show abundant pSTAT5-ir (Fig. 2r-t). These nuclei contain the dopaminergic neuron populations responsible for the inhibitory feedback control of hypophyseal PRL release[76]: the tuberoinfundibular (TIDA) and tuberohypophyseal (THDA) neurons in the dorsomedial and rostral Arc[77], and the

periventricular hypophyseal neurons (PHDA) in the Pe[78]. In fact, it has been shown in female rats that PRL feedback signal in these dopaminergic populations is conveyed through the Jak/STAT pathway, specifically through STAT5b[79]. Therefore, our results in the Arc and Pe likely reflect the (expected) occurrence of the same feedback mechanism in male mice.

Studies in lactating rodents ascribe an additional role of PRL in the regulation of energy balance and food intake[6]. This physiological process is controlled by a complex neural network involving several hypothalamic nuclei and different neurochemical signals[80]. According to our findings, some of the hypothalamic centres belonging to this network display responsiveness to PRL in the male mouse brain, namely the Arc, the VMH, the DM and the Pa. Taken together, our findings indicate that PRL might be involved in the control of food intake not only during motherhood, but also in non-lactating females and males, under certain physiological circumstances concurrent with high levels of circulating PRL (e.g. acute stress[81,82]).

Prolactin also plays an important role in the modulation of anxiety and stress. Concerning anxiety, studies in female rats indicate that PRL has anxiolytic properties when administered i.c.v. or i.v., whereas chronic i.c.v. antisense blocking of the PRLR expression results in increased anxiety[83]. In contrast to females, PRL has a weak anxiolytic effect in males[83]. This intersexual difference might be related to our findings of a sexually dimorphic responsiveness to PRL in the central structures directly involved in the regulation of anxiety and emotionality, the Ce and the anterodorsal BST [84]. In these nuclei, males are devoid of pSTAT5-ir (Fig. 1) but show PRLR mRNA transcript (Fig. 1 and Supplementary Fig. 2d-e) whereas females show quite abundant immunostaining, especially during pregnancy and lactation [4].

Concerning stress modulation, central inhibition of the expression of PRLR by i.c.v. antisense blocking results in an enhanced activation of HPA axis[83] in male and female rats, thus demonstrating a downregulation of the stress response by PRL. The presence of pSTAT5-ir in the Pa of males (in equivalent levels to females, see Figs. 2m, 2n and 3) suggests that this modulatory effect of PRL on

the reactivity of the HPA axis, would probably take place through PRL action on corticotropin-releasing factor (CRF) or vasopressin (AVP) cells [85]. In fact, PRL is a well-documented regulator of the function of magno- and parvocellular vasopressinergic neurosecretory cells at the level of the hypothalamic Pa [86–88]. Although effects of PRL on these parvocellular AVP cells has been proven to be mediated by ERK/MAPK pathway [88], our data in males and females suggest that the Jak/STAT5 signalling cascade can also be involved. This would imply a non-dimorphic regulatory role of PRL on the stress response (in addition to its role on anxiety).

Conclusions

This work characterizes the distribution of PRL responsiveness in the male mouse brain and its dependence on circulating PRL levels, sexual dimorphism and gonadal steroid regulation. Male mice display specific patterns of PRL-derived signalling only in the presence of high levels of circulating PRL. These patterns comprise mainly hypothalamic nuclei and some telencephalic sites, and are also more limited in extension and density than the equivalent patterns in female mice, evidencing a clear sexual dimorphism in favour of females. Furthermore, PRL-derived signalling in the male brain is regionally dependent on testosterone input, indicating a reciprocal regulation between both hormones. This work confirms that PRL exerts central actions in males, suggesting a role of the "maternal hormone" in the regulation of male sexual behaviour, PRL release feedback control, as well as in regulation of stress response.

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FIGURE LEGENDS

Figure 1. Mapping of pSTAT5 immunoreactivity and prolactin receptor expression in the brains of male and female mice

Semi-schematic camera lucida drawings of coronal sections of the mouse brain showing the distribution patterns of pSTAT5-ir in female and male mice (left side). and the pattern of PRLR expression in the brain of an adult male mouse specimen (right side), as determined by PRLR expression data obtained from the Allen Brain Institute (© 2004 Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: mouse.brain-map.org), experiment 72340223 (http://mouse.brain-map.org/experiment/show/72340223). In the left side, pink dots represent pSTAT5 expression exclusive of ovariectomized, steroid-primed females, whereas dark blue dots encode overlapping expression of pSTAT5-ir in both the female and male specimens. Black dots in the right represent PRLR expression in the male mouse brain. Each dot represents approximately 4 cell nuclei labelled for pSTAT5 or equivalent number of cells expressing the PRLR. Shaded areas illustrate the counting frames designed for the AVPe/VMPO region (Fig. 1a), the Pa (Fig. 1e), and the Arc, VMHvl, VMHc and VMHdm, DM and MePD (all frames in Fig. 1f) as part of the quantitative analysis performed in this work (see below). Approximate distance to bregma is enclosed for each section.

Figure 2. Representative examples of pSTAT5 immunoreactivity in the brain of female, intact male and castrated male mice

Photomicrographs illustrating pSTAT5 labelling in representative brain sections in an ovariectomized, steroid-primed female supplemented with PRL (I), an intact, PRL-supplemented male (II), a castrated, PRL supplemented male (III) and a intact control male lacking PRL supplementation (IV). Sections correspond to the preoptic hypothalamus (A), the paraventricular region in the anterior hypothalamus (B), the tuberal hypothalamus (C), the caudal septum (D) and the medial posterior amygdala (E). The approximate distance to bregma is indicated in each section. Scale bars correspond to 100 µm.

Figure 3. Quantitative analysis of pSTAT5 immunoreactivity in selected brain regions of female and male mice

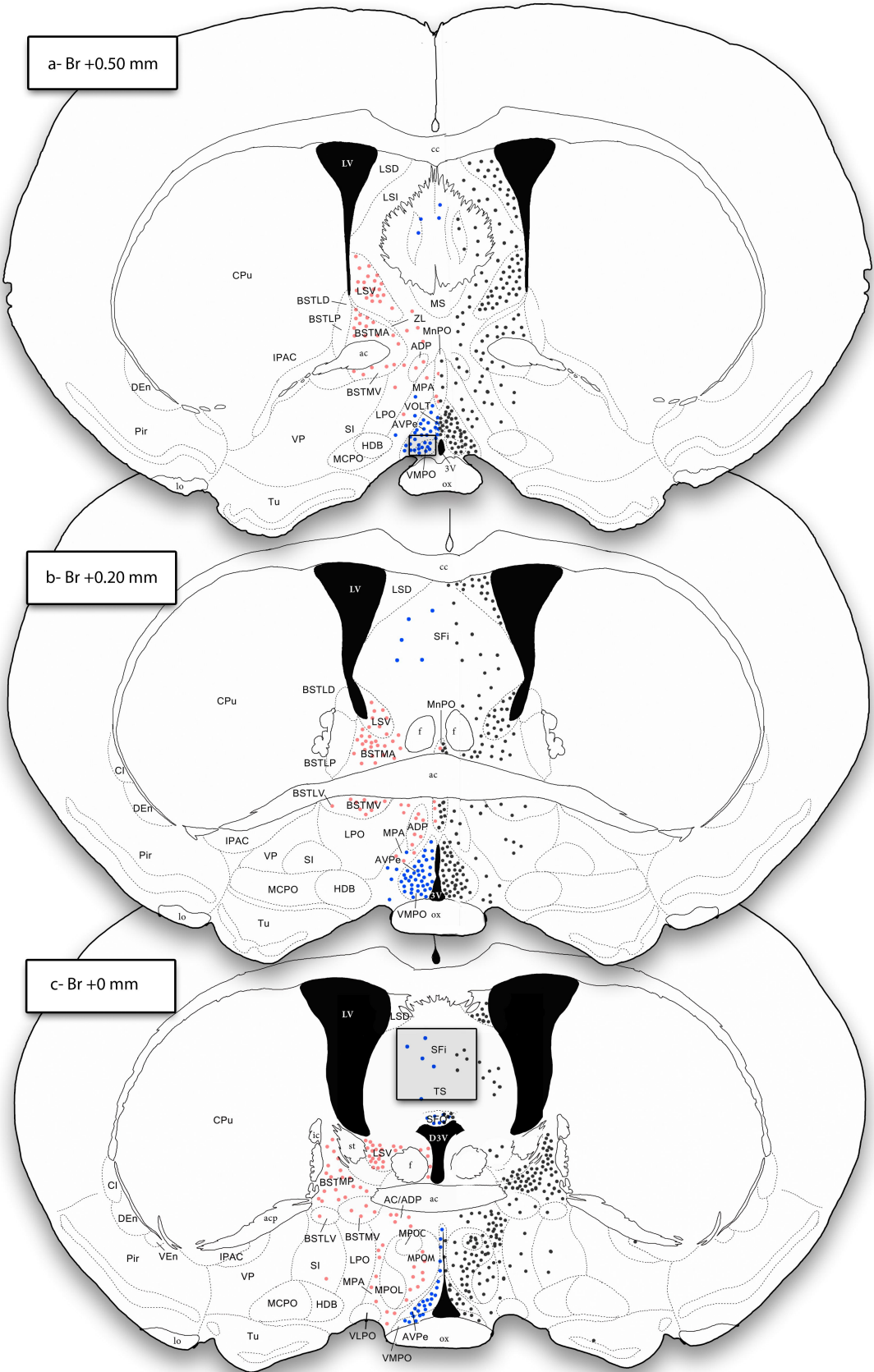
Assessment of pSTAT5-ir density (pSTAT5-positive cell nuclei/mm^2) in the major brain regions with expression of pSTAT5-ir in both male and female mice. Bar histograms show mean interhemispheric pSTAT5-ir density ± SEM in gonadally-intact, vehicle-treated males (Male+PRL; n=6; black) and ovariectomized females treated with estradiol and progesterone (Female+PRL; n=6; grey). Counting frames for each of the analyzed nuclei are included in Fig.1. Statistical analysis was applied independently to each brain region (independent t-test for parametric data or Mann-Whitney test for non-parametric data, see Results). *$P \leq 0.05$; **$P \leq 0.01$; (*) $P \leq 0.06$.

Figure 4. Effect of testosterone withdrawal in pSTAT5 immunoreactivity of the male mouse brain

Assessment of pSTAT5-ir density (pSTAT5-positive cell nuclei/mm^2) in gonadally-intact male mice and castrated male mice within the major brain regions showing pSTAT5 expression in the male mouse brain. Bar histograms show mean interhemispheric pSTAT5-ir density ± SEM in intact, PRL-supplemented males (Males+PRL; n=6; black) and castrated, PRL-supplemented males (Castrated+PRL; n=6; white). Counting frames for each of the analyzed nuclei are enclosed in Fig. 2.

1 Statistical analysis was applied independently to each brain region (independent t-test for

2 parametric data or Mann-Whitney test for non-parametric data). *$P \leq 0.05$; **$P \leq 0.01$.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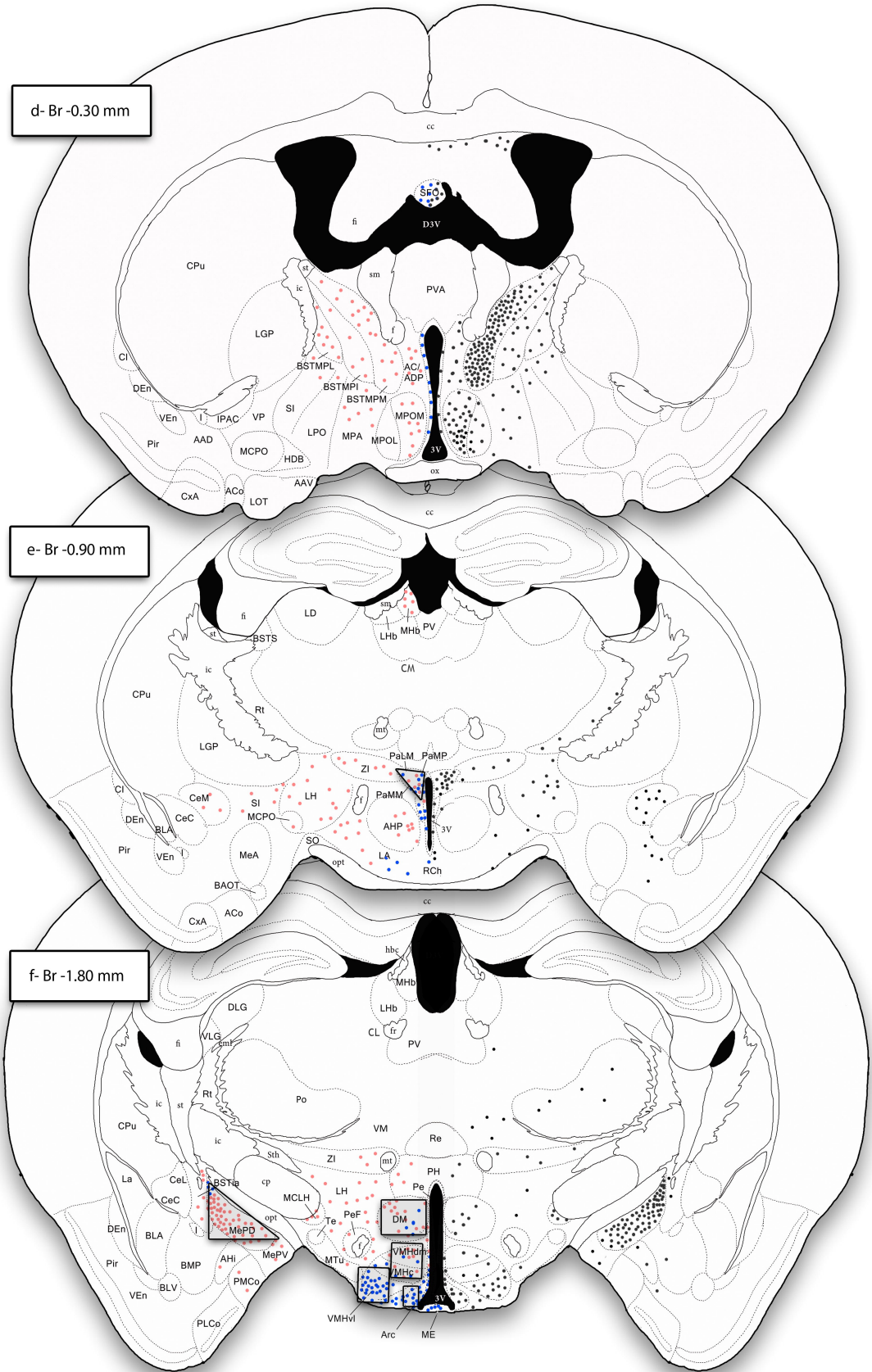
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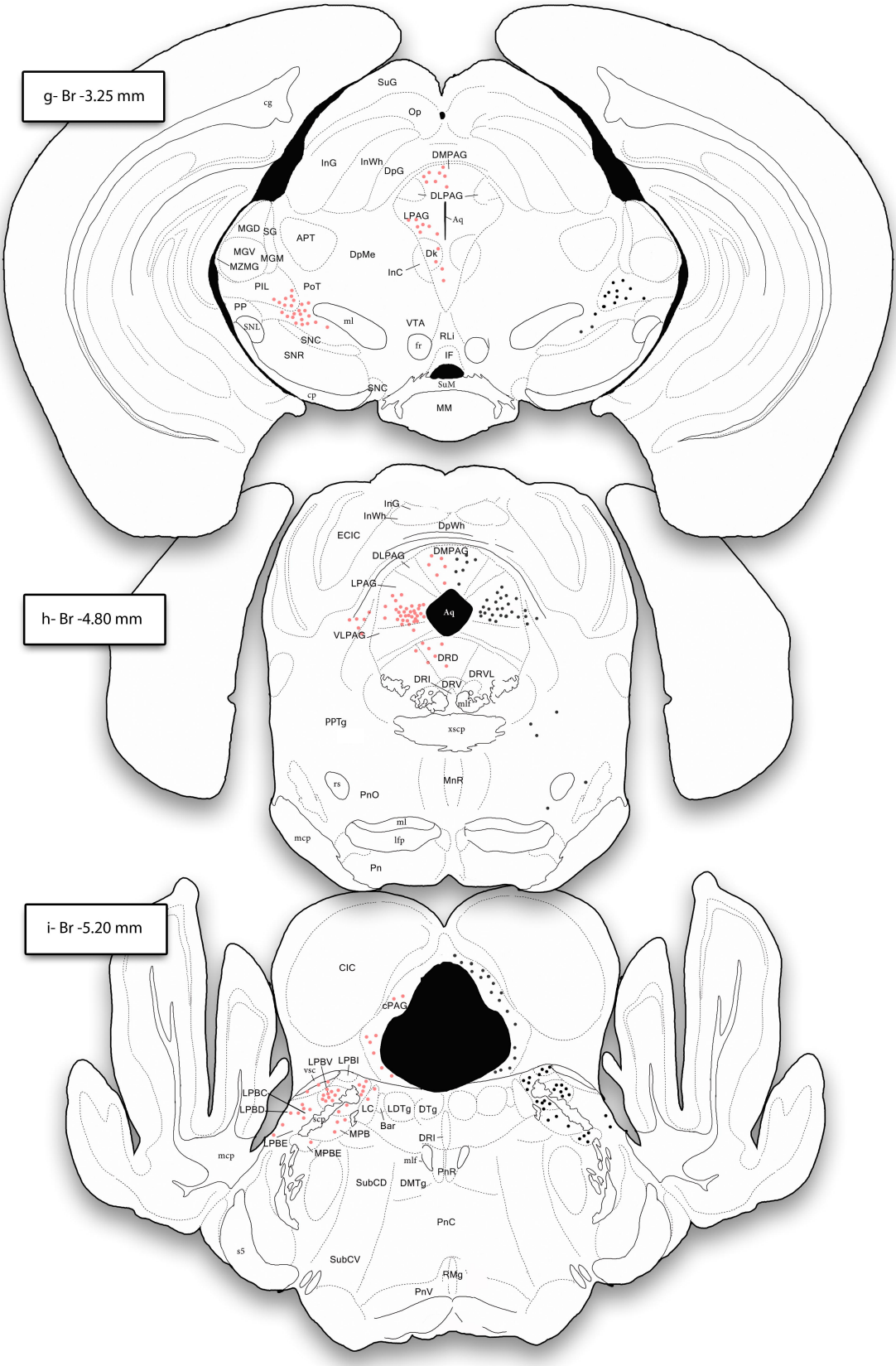
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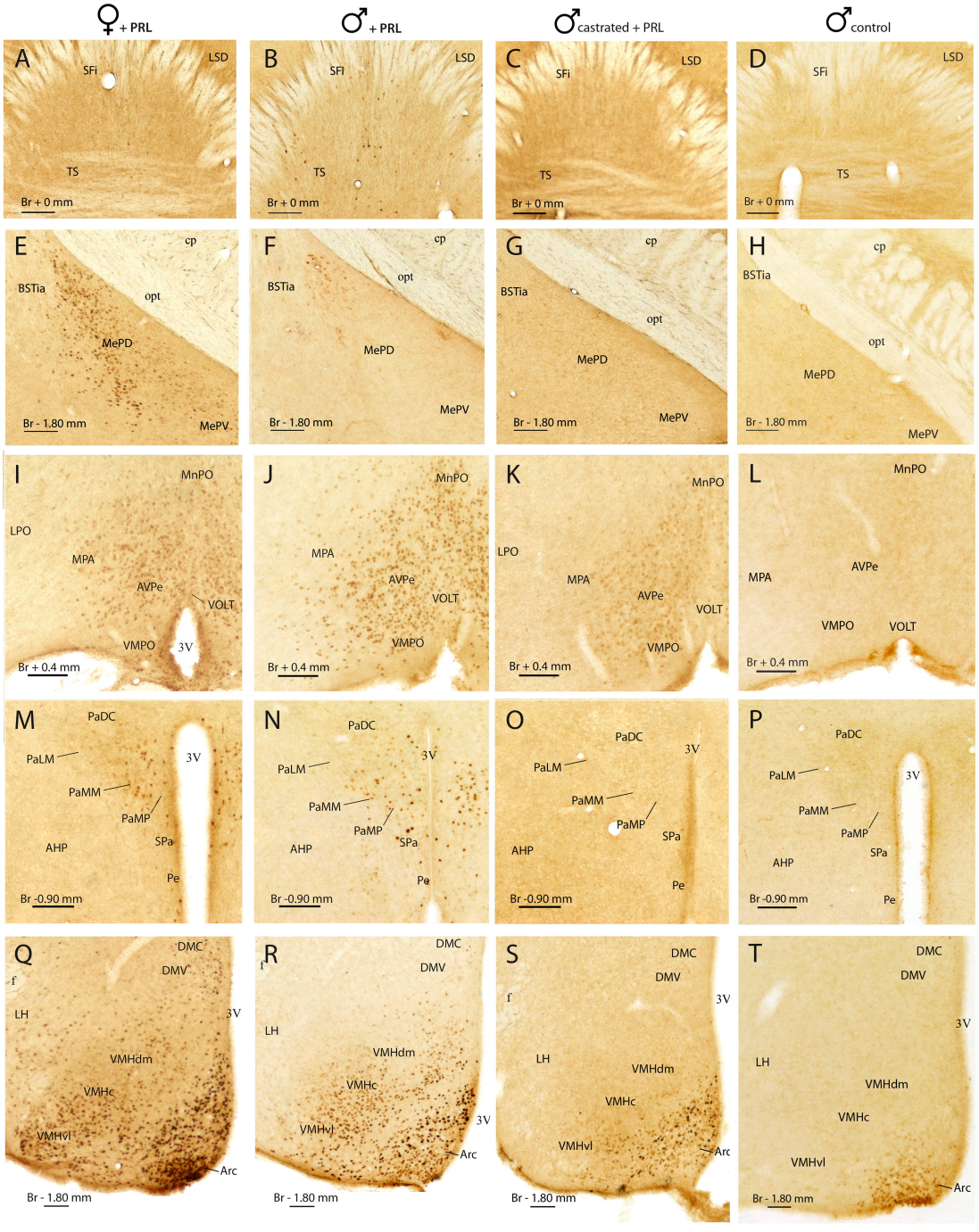
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VMHc
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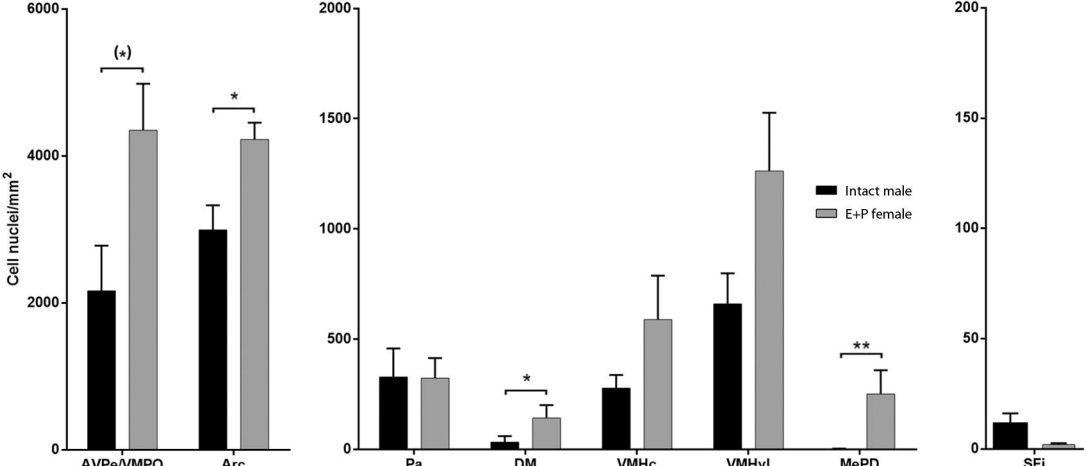
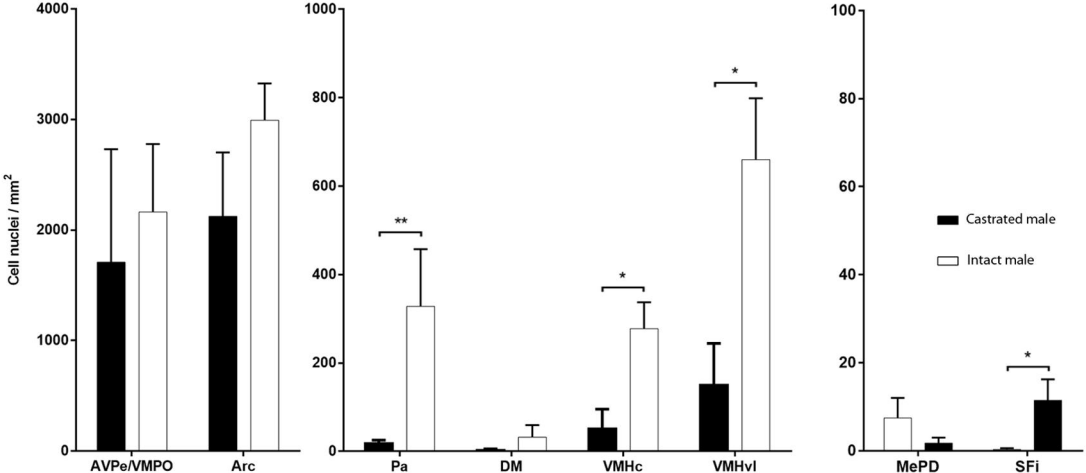
Arc
Br - 1.80 mm
R
DMC
DMV
f
LH
VMHdm
VMHc
3V
VMHvl
Arc
Br - 1.80 mm
S
DMC
DMV
f
3V
LH
VMHdm
VMHc
VMHvl
Arc
Br - 1.80 mm
T
DMC
DMV
3V
LH
VMHdm
VMHc
VMHvl
Arc
Br - 1.80 mm


Cell nuclei/mm2
6000
4000
2000
0
(*)
*
AVPe/VMPO
Arc
2000
1500
1000
500
0
*
**
Intact male
E+P female
Pa
DM
VMHc
VMHvl
MePD
200
150
100
50
0
SFi


Cell nuclei / mm²
4000
3000
2000
1000
0
AVPe/VMPO
Arc
1000
800
600
400
200
0
**
*
*
Pa
DM
VMHc
VMHvl
100
80
60
40
20
0
*
MePD
SFi
Castrated male
Intact male